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Research Article

Zasp/Cypher internal ZM-motif containing fragments are sufficient to co-localize with α-actinin—Analysis of patient mutations

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ABSTRACT

Z-band alternatively spliced PDZ-containing protein (ZASP/Cypher) has an important role in maintaining Z-disc stability in striated and cardiac muscle. ZASP/Cypher interacts through its PDZ domain with the major Z-disc actin cross-linker, α-actinin. ZASP/Cypher also has a conserved sequence called the ZM-motif, and it is found in two alternatively spliced exons 4 and 6. We have shown earlier that the ZM-motif containing internal regions of two related proteins ALP and CLP36 interact with α-actinin rod region, and that the ZM-motif is important in targeting ALP to the α-actinin containing structures in cell. Here, we show that the ZASP/Cypher internal fragments containing either ZM exon 4 or 6 co-localized with α-actinin in cultured myoblasts and nonmuscle cells. Fragments of 130 residues around the ZM-consensus were sufficient for localization, which is similar to our previous results of ALP. Moreover, ZASP/Cypher protein interacted directly with the α-actinin rod and competed with ALP in binding to the rod. During the inhibition of stress fiber assembly ZASP/Cypher and α-actinin co-localization could be partially disturbed, suggesting that ZASP/Cypher is bound to α-actinin mainly when α-actinin is localizing in stress fibers. Many point mutations found in cardiomyopathy patients are located in the internal region of ZASP/Cypher. However, we found no evidence that human patient mutations in the internal domain would affect the ZASP/Cypher co-localization with α-actinin, or that the mutations would destabilize the ZASP/Cypher protein.

Introduction

The cytoskeleton of muscle cells is organized into sarcomeres [1], which are held together through Z-discs, where actin filaments from adjacent sarcomeres are connected via α-actinin and many accessory proteins. α-Actinin is an antiparallel homodimer, composed of an N-terminal actin binding domain (ABD), a central rod region, and two pairs of C-terminal EF-hands [2]. A group of PDZ-LIM proteins localize at the muscle Z-disc and interact with α-actinin. This group is composed of Z-band alternatively spliced PDZ-containing protein (ZASP/Cypher/Oracle) [3–5], actinin-associated LIM protein (ALP) [6,7], C-terminal LIM domain protein (CLP36/Elfin/hCLIM1) [8–13], and Enigma homology

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protein (ENH) [14,15]. Also Enigma localizes at the Z-lines [16], but there is no evidence of the interaction to α-actinin so far. Reversion-induced LIM protein (RIL) and Mystique/Pdlim2 are also members of this group, but they are expressed only in nonmuscle cells [17–22]. Of these proteins, ZASP/Cypher, ALP and CLP36 have a 26-residue conserved sequence in the internal region between PDZ and LIM domains, named ZASP/Cypher-like motif (ZM). Together, these seven PDZ-LIM proteins form the Enigma family. Both PDZ and LIM domains are conserved and well-characterized folds [23,24], but the structure and function of the ZM-motif are unknown.

The role of the Z-line α-actinin-binding PDZ-LIM proteins in regulating the highly specialized contracting machinery of the muscle cell is not completely understood. The expression of ZASP/Cypher is restricted to muscle tissues [3,5]. ZASP/Cypher deficient mice die with severe disruption of sarcomeres in striated muscle [25]. The ZASP/Cypher mice phenotype implicates an indispensable function for ZASP/Cypher, which other Z-line PDZ-LIM proteins cannot compensate, in contrast to the milder ALP knockout phenotype [26]. The ZASP/Cypher knockout study emphasizes the role of ZASP/Cypher in mature contracting muscle [25] as a mechanical stabilizer or as a signaling component required for regeneration. Mouse ZASP/Cypher, as well as ALP, is also shown to co-localize with α-actinin already at the earlier stages of myofibrillogenesis [27,28].

ZASP/Cypher has one N-terminal PDZ domain and three LIM domains at the C-terminus. Several splice variants of ZASP/Cypher exist both in human and mouse [29,30]. For instance, ZASP/Cypher variant-1 does not have LIM domains, and a part of the internal region is either encoded by the ZM-motif containing exon 4 or exon 6. The PDZ domain of ZASP/Cypher recognizes the C-terminus of α-actinin [3,25], and the structure of the ZASP/Cypher PDZ implicates that all PDZ domains of the whole Enigma family would have similar mechanism of interaction with the C-terminus of α-actinin [31]. Earlier, we have shown that ALP and CLP36 have two interaction sites with α-actinin [32]. The PDZ domain recognizes the C-terminus in similar manner as ZASP/Cypher, and the internal region interacts with the rod region α-actinin. Our hypothesis was that the internal interaction site with α-actinin exists also in ZASP/Cypher. A previous study has shown that the internal part of ZASP/Cypher localizes to the Z-lines in cardiomyocytes [25]. Interestingly, point mutations in the internal region of ZASP/Cypher have been linked to certain cardiomyopathies and muscle dystrophies [30,33].

In this paper, we showed that internal ZM-motif containing fragments of ZASP/Cypher co-localized with α-actinin in cultured cells in similar manner as ALP and that ZASP/Cypher variant-1 interacted directly with the α-actinin rod region. We also found that ZASP/Cypher and α-actinin co-localization can be released after stress fiber disassembly, suggesting that in cell ZASP/Cypher is bound to α-actinin mainly when α-actinin is linked to filamentous actin. We could not detect any reproducible differences either in localization or in stability in the mutant ZASP/Cypher proteins compared to the wild type.

Materials and methods

Generation of human ZASP/Cypher constructs

Internal fragments of ZASP/Cypher exon 6 (residues 108–276) (AJ133767) and ZASP/Cypher exon 4 (112–298) (AB014513) were amplified from human skeletal muscle cDNA library (Matchmaker, BD Biosciences, Clontech) and cloned into pEGFP-NI vector (Clontech, BD Biosciences) using BglII-BamHI cloning site. These were used as templates to generate shorter fragments. ZASP/Cypher variant-1 wt (ENST00000310944) and K136M mutant in pEGFP-C1 vector were generous gifts from Matteo Vatta (Baylor College of Medicine, Houston, USA). The actual amino acid composition of exon 5 (residues 109–115) in variant-1 is VVNSPAN, which is different from the Ensemble transcript. The ZASP/Cypher variant-1 GFP-construct was used as a template in cloning variant-1 to modified pET24d vector (Novagen, Merck Biosciences, Schwalbach, Germany) where an N-terminal His_6-tag is followed by a tobacco etch virus protease recognition site [34]. The remaining ZASP/Cypher internal region point mutations described in Vatta et al. [30] and Selcen and Engel [33] were generated using QuikChange Site-Directed Mutagenesis (Stratagene Cloning Systems). ZASP/Cypher constructs generated for this study are listed in Table 1. Detailed information of α-actinin 2 rod (contains spectrin repeats 1–4) (274–746) and EF3-4 (the last pair of C-terminal EF-hands) (821–894) constructs used in this study are described elsewhere [32]. An internal fragment of ALP (residues 107–273) was cloned in modified pET24d as earlier [32].

Cell culture

Chinese hamster ovarian (CHO-K1) cells (ATCC CCL-61, American Tissue Culture Collection, Manassas, VA) and mouse myogenic C2C12 (ATCC, CRL-1772) cells were cultured and transfected with 4 μg of DNA on 6-well plates using Lipofectamine 2000 (Invitrogen) as described earlier [32].

In inhibitor experiments, transfected cells were allowed to attach on fibronectin coated coverslips [32] on 6-well plates for 30 min in culture media without serum and antibiotics. 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7) (I2764, Sigma-Aldrich) dissolved in 100% dimethylsulphoxide (D2650, Sigma-Aldrich) was added at 30 μM final concentration. After 60 min, inhibitor was removed by washing the cells twice with the serum- and antibiotic-free media. At selected time points, cells on coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and 0.5% Triton X-100. Samples were stained with antibodies and mounted with Vectashield-mounting media (H-1000, Vector Laboratories, Burlingame, CA, USA).

Antibodies used in immunofluorescence

α-Actinin was stained with a monoclonal α-actinin antibody (A5044, Sigma, 20 μg/ml) in CHO and C2C12 cells. Also a